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PCR Amplification of DNA Extracted from Formalin-Fixed Tissue †

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= Abstract =**In this study we try to confirm whether the DNA from formalin-fixed tissue be suitable for forensic application such as individual identification or examination of medicolegal evidences using PCR. Fresh placenta were taken from 18 different women and DNA was extracted . Some portion of placenta were fixed in 10% buffered neutral formalin for 24 hour and DNA was extracted. Two loci of D1S80 and D17S30 were used for PCR amplification. The amplification pattern were identical between fresh tissue and formalin fixed tissue suggesting that the formalin fixed tissue can be used for medicolegal situation. But the DNA from formalin seemed to be degraded as the fixation time prolonged.**

Key Words: *Formalin-fixed tissue, PCR*

INTRODUCTION

The development of DNA fingerprint (Jeffreys *et al.* 1985) in forensic medicine is making an increasingly important contribution to individual identification and examination of medicolegal evidences(Kanter *et al.* 1986). A limitation of DNA fingerprint has been its reliance on fresh tissue as a source of DNA. However recent investigation have shown that DNA can be recovered from neutral buffered formalin fixed tissue and that it is suitable for PCR amplification.

However, researchers have had variable success(Impraim *et al.* 1987; Wright and Manos 1990). Investigators identified the fixation process as the primary source of the reduction in size of extractable DNA(Goelz *et al.* 1985). The DNA extracted from fixed tissued has been reported to range in size from 10 to 24,000 base pair (bp). In two studies, the size of DNA fragments from tissues fixed in 10% buffered neutral formalin ranged from 100 to 1,500bp(Goelz *et al.* 1985) and 4,000-9,000bp(Impraim *et al.* 1987). In some cases DNA that is too degraded for Southern or other hybridization analysis may be suitable for PCR-based methods.

It is of value to researchers to know which tissue processing conditions are most conductive to subsequent PCR. This knowledge can be incorporated into protocols for prospective studies or for identification of appropriate samples for retrospective analysis. Although our interest is mainly in retrospective medical studies, determining the best methods for preparing and storing specimens for DNA analysis is an urgent need for scientists in other disciplines as well.

In this study, different approaches were used to recover DNA. Observations on the effect of fixation time on the recovery of DNA

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were also done. The effect of fixation was measured by the ability of a treated tissue to serve as template for the amplification of DNA fragments that ranged from 323-771bp in length.

MATERIALS AND METHODS

1. Samples

Fresh placenta obtained at routine delivery room was divided into about 1X1X1cm blocks weighing 1gm. Fresh samples were frozen in liquid nitrozen and stored at -70°C. The remaining tissue was fixed for 24 hour in 10% buffered neutral formalin.

Other two set of samples with variable fixation time were prepared to see the effect of fixation time in formalin. The fixation time ranged from 1 day up to 2 weeks. Following extraction method was same.

2. Extraction of nucleic acids

Fresh tissue DNA was prepared as described by Kunkel *et al.* (1982). Spectrophotometer measurement at 260nm and 280nm were used to calculate the DNA yield and the degree of protein contamination of samples. Quantitative analysis of the molecular weight of DNA was assessed eletropholetically by running 5 μg aliquots of extracts in submerged 0.8% agarose gels and photography after ethidium bromide staining.

DNA extraction from formalin-fixed tissue were done in several way to improve the yield.

1) Usual DNA extraction from fresh tissue after thorough washing and mincing(Maniatis *et al.* 1989)

2) Pepsin treatment

0.5% pepsin treatment was done for 3 hour under 37°C shaking water bath before the process 1).

3) Sonication

Sonication was done under 30 μm, 20sec condition for four times before the process 1).

4) Thermal treatment

Minced tissue was heated 65°C for 6 hours and followed by usual process 1).

5) Other detergents tween 20 or NP 40 were added to the usual digestion solution and followed by the process 1).

3. PCR amplification

Amplification was done for two DNA loci, D1S80 and D17S30 as described by Lee(1991). Briefly describing, the primers in amplification of D1S80 locus were 5'-GAAACTGGCCTCC AAACACTGCCCGCCG-3' and 5'-GTCTTGTTG GAGATGCACGTGCCCCTTGC-3'. Two nanograms of genomic DNA were amplified in 25 μl of a reaction mixture containing 67mM tris-Hcl (pH 8.3), 6.7mM magnesium chloride, 16.6 mM ammonium sulfate, 10mM 2-mercaptoethanol, 170 μg/ml bovine serum albumin, 10% dimethyl sulfoxide, 2.5mM each of the deoxy forms of the dNTP mixture, 1.25 unit of Taq polymerase and 2 μM of each primer. After denaturation of the DNA at 95°C for 1min, annealing was done at 65°C for 1 min with an extension at 70°C for 8min and repeated for 30 cycles.

The primers in the amplification of the D17S30 locus were 5'-CGAAGAGTGAAGTGC ACAGG-3' and 5'-CACAGTCTTTATTCTTCA GCG-3'. The PCR was carried out in 25 μl of a reaction mixture containing 50 ng of genomic DNA, 0.5 μM of each primer, 300mM of each dNTP, 10mM Tris-Hcl(pH 8.3), 50mM Kcl, 2.5mM magnesium chloride and 1.25 unit of Taq polymerase. After denaturation for 5 min at 94°C, denaturation for 1min at 95°C and annealing for 1min at 55°C were carried out, followed by extension for 8min at 72°C and repeated for 30 cycles.

The polymorphism of the amplified products was analysed by silver staining after electrophoresis on polyacrylamide gels.

RESULTS

Above 5 methods of extracting DNA from formalin fixed placenta tissue showed variable degree of results. The 4th method using thermal treatment showed most satisfiable results than the others. The DNA extracted by sonication and pepsin digestion was less tham 2Kb in

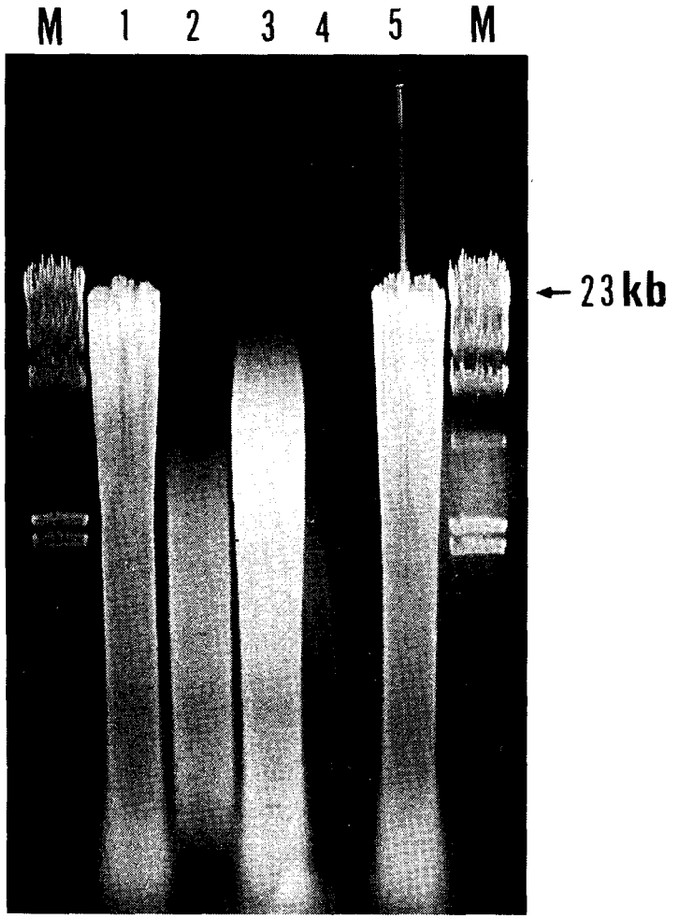

Fig. 1. DNA pattern isolated from formalin-fixed tissue for 1 day fixation by thermal treatment (lane 1), pepsin digestion (lane 2), additive detergents (lane 3), sonication (lane 4) and conventional extraction method (lane 5). M; size standards from lambda DNA digested with Hind III.

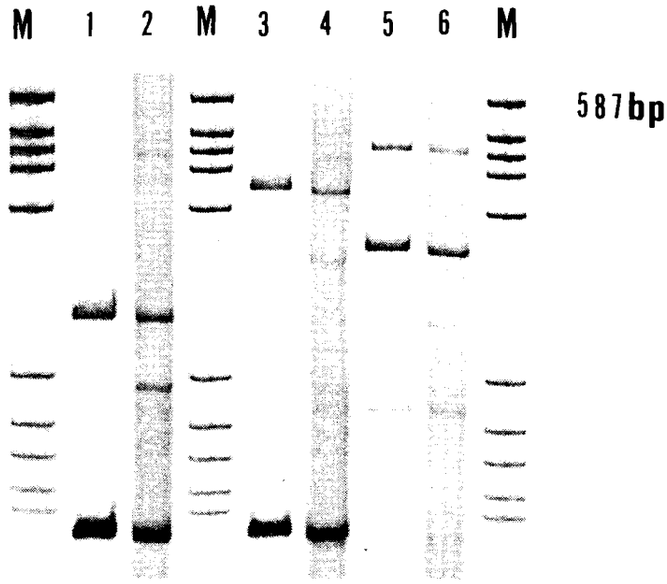

Fig. 2. Expression of D17S30 amplified fragments between fresh tissue (lane 1, 3 and 5) and formalin-fixed tissue (lane 2, 4 and 6). M; size standards from pBR322 DNA digested with Hae III.

agarose gel electrophoresis. The either conventional extraction or additive detergent extraction resulted in marked decrease of extraction yield, though the DNA quality was as good as those of thermal treatment. We obtained a spoolable DNA only in the thermal-treated extraction and the isolated DNA was of relatively high molecular weight.

Once the spoolable DNA was obtained, the DNA quality was satifiable to accomodate PCR amplification showing the same electrophoresis band pattern as that of fresh tissue.

With the additional purification of the sample using spun column to improve the amplification efficiency there was little or no significant improvement in the amplification with regard to the spun-purification.

DISCUSSION

We have shown that DNA can be extracted from formalin-fixed tissue, but the process must be different from that used in case of fresh tissue. Many authors have used different methods for extraction of DNA. Goelz *et al.* (1985) used simple prolonged digestion, meanwhile Dubeau *et al.* (1986) used complicated method yielding DNA free from the contaminants associated with the former procedure. According to Feldman(1973), the reaction of formaldehyde with mono- and polynucleotides proceeds in two stages: rapid but reversible hydroxymethylation of imino and amino groups on bases, then slow formation over a period of days, of methylene bridges between bases. Thus altered physical property by crosslinking seems to resist enzyme digestion, such as proteinase K or restriction enzyme. This is consistent with our result that we cannot acquire

satisfactory results with ordinary method. The reactions between formaldehyde and native DNA are enhanced by an increase in the reaction temperature. At room temperature the double helix of DNA is present as two complementary polynucleotide chains linked together by base pair hydrogen bonding. On heating to 65°C, however, these bonds begin to break and eventually at about 90°C, two single stranded molecules are produced. In this denatured state, formaldehyde reacts rapidly, through hydroxymethylation, and this inhibits reannealing on cooling. Using these properties we can obtain spoolable DNA from formalin-fixed tissue. Rare other people reported improved data on heating the sample(Jackson *et al.* 1990).

Generally it is known that DNA is fragmented on fixation, but the size of the fragmented DNA does not appear to be the sole predictor of successful PCR amplification. there are factors that limit the size of the amplification produsts, perhaps the frequency of cross-linking moieites present in the DNA. That formalin fixation results in some physical alteration of DNA is also apparent from the presence of medium molecular weight DNA in nondigested sample.

The result that there was no advantage provided by additional purification of the fixed sample may be correlated with the high efficiency of the primers used in this study. Amplification with primers that are degenerate or less efficient may be enhanced by additional purification of the target DNA. And older specimens or those fixed under suboptimal conditions, may require the use of primer pairs that generate smaller amplification products.

Observations on the effect of fixation time on the recovery of DNA also differed. Goelz *et al.* (1985) claimed that the quality of recovered DNA was not affected by the fixation time, whereas Dubeau *et al.* (1986) demonstrated a reduction in the amount of DNA extracted when the fixation time exceed 5 days. But according to this study, as the fixation time is extended, alterations to DNA will limit the available range.

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